

THE VITALITY OF FLUORESCENCE MICROSCOPIC INVESTIGATIONS CARRIED OUT WITH THE  
HELP OF THE FLUOROCHROMES OF ACRIDINE ORANGE AND 3,4 BENZPYRENE

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HELP OF THE FLUOROCHROMES OF ACRIDINE ORANGE AND 3,4 BENZPYRENE

[Following is the translation of an article by Ye.A. Lyapunova, S. B. Petrikevich and A. V. Zelenin, Institute of Molecular Biology, AN USSR, published in the Russian-language periodical Izvestiya AN SSSR, Seriya Biologicheskaya (Bulletin of the USSR Academy of Sciences, Biology Series), No 6, 1965, pages 928-932. It was submitted on 24 Nov 1964. Translation performed by Sp/7 Charles T. Ostertag Jr.]

It has been considered that one of the virtues of fluorescence microscopy is the feasibility of carrying out intravital investigations of cells and individual cellular structures. However, in the majority of cases opinions on the "vitality" are quite superficial. The condition of cells, treated with one or the other fluorochrome, can usually be judged based on their morphological picture and in some cases on the ability for multiplication (Robbins, Marcus, 1963) or on the ability for going through mitosis (Hill et al., 1959).

We made an attempt to obtain several additional concepts on the influence of fluorochroming of cells on their activity. As the main substance being investigated we selected acridine orange (2,8 bis-dimethyl-aminoacridine)--the fluorochrome which is used most widely in fluorescence investigations (Meysel, Gutkina, 1961). For a comparison we tested the action of 3,4 benzpyrene on the same cells. With its help it was possible to obtain the complete exposure of lipids in a living cell (Berg, 1951; Breyvis, 1957).

A study was made on the influence of acridine orange treatment of cells on the synthesis of proteins and nucleic acids in them and also on the mitotic activity of the culture.

The object of the investigation was amniotic epithelium (strain Fl) cells cultivated in vitro. The metabolism of protein and nucleic acids was judged on the basis of data from autoradiographic investigations, as this was described earlier (Zelenin, Lyapunova, 1964a). As a result of the tests it was established that acridine orange possesses a high toxicity in respect to a living cell and in a concentration of 2--3  $\mu\text{g/ml}$  completely suppresses the mitotic activity of a culture. It was revealed

that the toxicity of acridine orange is connected with the specific inhibition of protein metabolism (Zelenin, Lyapunova, 1964) and that the stated fluorochrome also possesses a depressing influence on the synthesis of nucleic acids, primarily RNA (Zelenin, Lyapunova, 1964B).

In table 1 we present the results of the 24 hour action of various concentrations of AO on the mitotic activity of a culture and the synthesis of protein in it. It can be seen from the table that acridine orange possesses a sharply depressing influence on the cell, beginning with a concentration of 1  $\mu\text{g/ml}$  and higher. When AO is used in a concentration of around 0.5  $\mu\text{g/ml}$  the synthesis of protein, though still somewhat inhibited, still in general takes place quite actively. The stated concentration of AO has little influence on the general condition of the cells. This is testified to by the high mitotic activity of the culture (table 1).

It follows from what has been said that fluorescence microscopic investigation of cells, treated with acridine orange in a concentration of 0.1--0.5  $\mu\text{g/ml}$  ( $3.3 \cdot 10^{-7}$ -- $1.67 \cdot 10^{-6}$  moles), is almost completely intravital (vital). In preparations fluorochromed with AO in such concentrations, the nucleus is clearly dimmer than the other parts of the cell and there are quite bright nucleoli and lysosome-like cellular structures -- red cytoplasmic granules -- which have absorbed large quantities of acridine orange (Zelenin, 1965). In spite of the fact that the pictures obtained during such an investigation are not very bright, it is quite possible to use it for obtaining interesting information about the condition of the nucleoli and (to a certain degree) also DNA. It is possible to obtain general concepts of the vital activity of cells based on the formation of red cytoplasmic granules by the cells. As Robbins et al. (1964) demonstrated recently, the fact that the cell concentrates acridine orange in the form of cytoplasmic granules testifies to the normal functioning of the main mechanisms of energetic metabolism in it.

Usually higher concentrations of AO (10--20  $\mu\text{g/ml}$ ) are used in the practice of fluorescence microscopic investigation. As was already mentioned, acridine orange even in a concentration of 2--3  $\mu\text{g}$  per 1 ml completely suppresses the synthesis of protein in the cells, as a result of which they die after a certain period of time. However, a short duration action by AO in the stated concentrations is endured by the cells comparatively well. They completely preserve the ability for granule formation. In the course of the first hours after the onset of action by AO mitosis takes place normally in the cell. As special tests showed (table 2), at an AO concentration of around 20  $\mu\text{g/ml}$ ,  $\text{H}^3$ -thymidine is actively incorporated in the nucleus of the cell. This testifies to the feasibility of DNA synthesis in the cell, and also to the harmlessness of the processes of oxidative phosphorylation and respiration.

In our opinion fluorochroming of live cells with AO in a concentration of 10--20  $\mu\text{g/ml}$  ( $3.3 \cdot 10^{-5}$  --  $6.6 \cdot 10^{-5}$  moles) can be expediently called supravital. Such a fluorochroming has a number of advantages in comparison with vital. Mainly it produces a considerably brighter fluorescence microscopic picture and also opens wide possibilities for the investigation of DNA and nucleolus RNA. In particular, as our experiments showed, with the help of the supravital fluorochroming it was possible to obtain important information concerning the action of antibiotics of the actinomycetes D type on DNA (Zalmanzon et al., 1965). During supravital fluorochroming the preparations are already obtained as a result of a brief (several minutes) treatment with AO. This is quite necessary when investigating preparations made up of smears and imprints of cells. What has been said makes it understandable why supravital investigation is the most prevalent type of fluorescence microscopic study of a living cell. Such an investigation is very simple but at the same time produces considerable information about the cell. All the same it is necessary to keep in mind that the investigator is not dealing with a living cell, but only with a cell which has preserved a number (maybe the majority) of features of a living cell.

In certain fluorescence microscopic investigations the live cells are placed in a solution of acridine orange with a higher concentration than during supravital fluorochroming (100--200  $\mu\text{g/ml}$  --  $3.3 \cdot 10^{-4}$  --  $6.6 \cdot 10^{-4}$  moles). The cells are killed and a fluorescence microscopic picture emerges which is typical for the fluorochroming of a fixed cell (green or yellow-green nucleus, diffuse red cytoplasm). Fluorochroming, during which the death of the live cell sets in, should apparently be called pseudovital. To a certain degree such fluorochroming is equivalent to the fluorochroming of fixed preparations, however the investigations here are not sufficiently rigid. In particular the pH of the fluorochrome is not taken into consideration and, as is known, it has great importance for a correct exposure of nucleic acids by the fluorescence microscopic method (Armstrong, 1956; Schummelfeder et al., 1957; Vorotnitskaya et al., 1963). Therefore the use of those concentrations of acridine orange which might lead to the pseudovital fluorochroming of the cell is clearly not suitable and cannot be recommended.

It is necessary to stress that the proposed classification for the types of fluorescence microscopic investigation of living cells is only schematic. The concrete concentrations of AO quoted, at which the investigations bear a vital or supravital nature, are accurate only relative to the amniotic cell culture used in our experiments. Available data in the literature (Hill et al., 1959; Robbins, Marcus, 1963) gives a foundation to the proposal that concentrations of AO, with the help of which it is possible to carry out vital investigations, may fluctuate somewhat even in respect to the cells of tissue cultures of various strains.\*

Experiments on the influence of 3,4-benzpyrene on cultural cells of amniotic epithelium (strain FL) showed that in comparison with acridine orange this substance is considerably less toxic; it turned out that the influence of benzpyrene even in a concentration of 55  $\mu\text{g/ml}$  for 24 hours is not reflected in the cellular synthesis of protein, determined by the incorporation of  $\text{S}^{35}$ -methionine (table 3). At the same time concentrations of this substance in 15--25  $\mu\text{g/ml}$  make it possible to obtain distinct fluorescence microscopic pictures. Though 3,4-benzpyrene in concentrations of 5--25  $\mu\text{g/ml}$  still exerts a certain influence on the cell, obviously interfering in the mechanism of mitosis (table 4), this action on its part is essentially insignificant. Therefore, at the present time the fluorescence microscopic exposure with the help of this fluorochrome can be considered intravital (vital). It is necessary however to make a reservation when using 3,4-benzpyrene in the practice of fluorescence microscopy. In no case should one forget its high degree of carcinogenesis.

#### Conclusions

1. Cytological and autoradiographic methods were used to investigate the degree of vitality of certain types of fluorescence microscopic investigation of living cells.
2. On the basis of tests, carried out with acridine orange fluorochrome, a classification is proposed for the types of fluorescence microscopic investigation: Vital (fluorochrome hardly reflected in the metabolism and viability of the cell); supravital (fluorochromed cell preserves the majority of features of a living cell, but not all of them, in particular such a treatment of cells with acridine orange leads to inhibition of protein synthesis); pseudovital (the cell dies as a result of the action of fluorochrome).
3. It was established that when working with a culture of cells of amniotic epithelium (strain FL), acridine orange in a concentration of 0.3--0.5  $\mu\text{g/ml}$  makes it possible to conduct a vital study, and in a concentration of 10--20  $\mu\text{g/ml}$  a supravital study of the cells. With the help of 3,4-benzpyrene in a concentration of 15--25  $\mu\text{g/ml}$  it is possible to make a vital investigation of the distribution of lipids in the same cells. The stated concentrations refer to cells which have been shielded from the action of light.

\*All the data cited in this article concerning the influence of AO and 3,4-benzpyrene relate to cells which were protected from the action of light during fluorochroming. It is necessary to keep in mind that exposure of the cells being fluorochromed can sharply raise the toxicity of fluorochromes as a result of their photodynamic action.

## Literature

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Table 1

Influence of a 24 hour action of A0 on the mitotic activity of a culture and the incorporation of labeled amino acids into its cells  
(summary data based on the results of 5 tests)

Concentration of A0, $\mu$ g/ml	Number of mitoses, % of control	Number of silver granules per equal area, % of control	Concentration of A0, $\mu$ g/ml	Number of mitoses, % of control	Number of silver granules per equal area, % of control
0	100	100	1.0	35	41
0.1	85	84	1.5	24	25
0.5	56	54	3.0	10	17



Table 2

Influence of AO on the incorporation of  $H^3$ -thymidine

Concentration of AO	Number of silver granules per 1 nucleus, in absolute figures	
	Control	1.5 hours after the onset of treatment with AO
18 $\mu$ g/ml	13.6	8.46

Table 3

Action of various concentrations of 3,4-benzpyrene on the incorporation of  $S^{35}$ -methionine

Concentration of 3,4-benzpyrene, $\mu$ g/ml	Number of silver granules per equal area occupied by cells, (in absolute numbers)	
	Test 1	Test 2
0	1332	4710
15	1428	3939
55	1387	3674

Table 4

Action of 3,4-benzpyrene on the mitotic activity of cells

3,4-benz- pyrene μg/ml	Number of mitoses in % to total number of cells, action of 3,4-benzpyrene - 24 hours				3,4-benz- pyrene μg/ml	Number of mitoses in % to total number of cells, action of 3,4-benzpyrene - 24 hours			
	Tests			Average		Tests			Average
	1	2	3			1	2	3	
0	3.04	3.29	2.69	3.0	35	2.07	1.94	2.16	2.06
5	3.52	3.87	3.69	3.7	45	2.79	1.48	1.68	2.0
15	3.48	3.80	3.21	3.5	55	2.57	1.76	1.75	2.0
25	3.50	3.63	2.61	3.25					